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The effect of temperature during culture enrichment on methanotrophic polyhydroxyalkanoate production

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ABSTRACT

Climate change and plastic pollution are likely the most relevant environmental problems of the 21st Century. Thus, one of the most promising solutions to remedy both environmental problems simultaneously is the bioconversion of greenhouse gases, such as methane (CH_4), into bioplastics (PHAs). However, the optimization of this bioconversion platform is still required to turn CH_4 biotransformation into a cost-effective and cost-competitive process. In this context, the research presented here aimed at elucidating the best temperature culture conditions to enhance both PHA accumulation and methane degradation. Six different enrichments were carried out at 25, 30 and 37 °C using different inocula and methane as the only energy and carbon source. CH_4 biodegradation rates, specific growth rates, PHA accumulations and the community structure were characterized. Higher temperatures (30 and 37 °C) increased the PHAs accumulation up to 30% regardless of the inoculum. Moreover, *Methylocystis* became the dominant genus (~30% of the total population) regardless of the temperature and inoculum used. This research demonstrated for the first time the fundamental role of temperature in increasing both the accumulation of PHAs and methane abatement during the enrichment of PHA cell-factories from methane, thus enhancing the cost-effectiveness of the process.

1. Introduction

Nowadays, the two most important environmental issues that our society is facing are Climate Change and Plastic Pollution. In this sense, novel industrial strategies are necessary to abate toxic greenhouse gasses (GHG) and to substitute current used plastic materials without compromising present economic and industrial development.

Methane (CH_4) is currently the second most important GHG (Desai and Harvey, 2010) and its atmospheric concentration continues to rise at a yearly rate of 0.2–1% mainly due to anthropogenic activities (agriculture, livestock, waste management and energy production) which has caused growing interest, from a political and a scientific point of view, in greenhouse gas abatement ((EPA), 2017). On the other hand, the massive usage of plastic products and poor management of waste disposal has resulted in microplastic contamination of bodies of water, which injures wildlife through entanglement or ingestion of plastics and through the toxic and carcinogenic effect of some polymers. Additionally, it has been demonstrated that plastics can make their way up the food chain, causing similar health threats to humans. This situation has encouraged an intensive research to find and produce biodegradable and non-toxic plastics or biopolymers, such as polyhydroxyalkanoates (PHAs) which could definitively substitute and end the use of pollutant plastics (Löhr et al., 2017; Pieja et al., 2017; Sigler, 2014).

PHAs are synthesized under nutrient-limited and carbon-excess conditions by different microorganisms (Pieja et al., 2017). The most common feedstock used for production of PHAs are glucose and fructose, but those carbon sources have a high price and entail a high market price of PHAs (4–20 € $KgPHA^{-1}$) (Koller et al., 2017). PHAs are currently industrially produced by nearly thirty corporations (Cantera et al., 2019). An innovative feasible alternative to enhance the economic sustainability of the degradation and valorization of CH_4 combined to the concomitant replacement of common plastics is the co-production of polyhydroxyalkanoates (PHAs) combined with the treatment of methane emissions (Cal et al., 2016; Pieja et al., 2017; Strong et al., 2016). Indeed, several studies have demonstrated that methanotrophs are a potential source of bioplastics, achieving PHA contents ranging from 20 to 60% (wt) using methane as feedstock (Cantera et al., 2019; Pieja et al., 2017). Methanotrophs are divided into two different groups according to the pathway for carbon

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assimilation a) type I (γ-proteobacteria) use the ribulose monophosphate (RuMP) pathway or b) type II (α-proteobacteria) use the serine pathway. Most of the studies reporting PHAs production using methanotrophic bacteria have been conducted with α-proteobacterial methanotrophs, since PHAs synthesis occurs in the serine cycle. Thus, the acetyl-CoA molecules produced in the serine cycle are transformed into PHAs via reactions catalyzed by the enzymes β-ketothiolase (phaA), acetoacetyl-CoA reductase (phaB), and PHAs synthase (phaC) (Cantera et al., 2019). Overall, type II methanotrophic bacteria such as *Methylocystis, Methylosinus* and *Methylocella* have been considered the main methanotrophic PHA producing genera under nutrient-limited conditions (i.e N-, P- or Mg-limitation) (García-Pérez et al., 2018; Helm et al., 2008; Myung et al., 2017; Rostkowski et al., 2013; Sundstrom and Criddle, 2015; Wendlandt et al., 2001; Zhang et al., 2017).

However, the cost-effective implementation of current biotechnologies for the biotransformation of CH_4 into bioplastics is still limited by the poor understanding of those factors that determine the selective enrichment of high-performance PHA producing methanotrophs. In this sense, different operational parameters such as pH, CH_4/O_2 ratio, or the concentration of sodium, copper or citrate have been reported as key factors for the enrichment of type II methanotrophs with the ability to synthetize PHA (Pieja et al., 2011; Scheutz et al., 2009; Semrau et al., 2013). However, there is still a need for studies assessing the effect of environmental factors such as temperature during culture enrichment of PHA producing methanotrophs. The use of an optimal temperature and the inoculum source have been reported as key strategies for the enrichment of microorganisms with the ability to synthetize PHAs using waste water and sugars as carbon sources (De Grazia et al., 2017). Thus, those inocula that favor the enrichment of type II methanotrophs, as well as the set temperature during enrichment could enhance this innovative biotechnology based on the transformation of methane into bioplastics. In this sense, *Sphagnum* mosses, which occur mainly in peat bogs, conifer forests and moist tundra areas, are described as the bryophytes with the highest richness of type II methanotrophs (Kip et al., 2011; Stępniewska and Kuźniar, 2014). Indeed, the extremophile environments that *Sphagnum* mosses inhabit, combined with the inherent presence of type II methanotrophs in their outer cortex, suggests their potential as a novel source of effective PHA-accumulating microorganisms (Kadouri et al., 2003; Ruiz et al., 2001; Zhao et al., 2007). Similarly, the stress caused on the metabolism of type II methanotrophs by an exposure to high temperatures might mediate an increase in PHA synthesis.

The study presented here aimed at systematically elucidating the influence of temperature (25 °C, 30 °C, 37 °C and 45 °C) during culture enrichment on the PHA synthetizing capacity, kinetics and stoichiometric characteristics, and population structure of microbial communities enriched using *Sphagnum* peat moss and a mixture of *Sphagnum* and activated sludge as inoculum.

2. Materials and methods

2.1. Mineral salt medium and inoculum

The MSM (a modified NMS medium Whittenbury et al. (1970)) was composed of ($g\,L^{-1}$): 2.25 $NaNO_3$, 0.1 $MgSO_4{\cdot}7H_2O$, 0.02 $CaCl_2{\cdot}2H_2O$, 0.68 KH_2PO_4, 6.14 $Na_2HPO_4{\cdot}12H_2O$, 1.3×10^{-3} $FeSO_4{\cdot}7H_2O$, 3.5×10^{-3} $MnCl_2{\cdot}4H_2O$, 1.5×10^{-3} $ZnSO_4{\cdot}7H_2O$, 0.04×10^{-3} $Na_2MoO_4{\cdot}2H_2O$, 0.04×10^{-3} $CuSO_4{\cdot}5H_2O$, 0.32×10^{-3} $CoCl_2$, and 0.2×10^{-3} H_3BO_3. Moreover, vitamins (biotin, nicotinamid, p.aminobenzoic acid and panthotenic acid) were added to stimulate the growth of sensitive methanotrophs.

All chemicals needed for MSM preparation were purchased from PANREAC (Barcelona, Spain), while CH_4 (≥99.5%) and O_2 (≥99.0%) were obtained from Abelló Linde S.A. (Barcelona, Spain). Poly [(R)-3-hydroxybutyric acid-co-(R)-3-hydroxyvaleric acid] (molar ratio 88/12, ≥ 99.99%) was purchased from Sigma-Aldrich® (St. Louis, MO, USA).

Fresh activated sludge from a denitrification-nitrification wastewater treatment plant (Valladolid, Spain) (López et al., 2018b) and *Sphagnum* peat moss from *Plantas Carnívoras*, (Madrid, Spain) were used as inocula for the enrichment of microorganisms able to degrade CH_4 and accumulate PHA. The *Sphagnum* peat moss was diluted 10 times in MSM prior inoculation.

2.2. Culture enrichment at different temperatures

A preliminary enrichment was performed batch-wise for 19 days at 25 °C in 1250 mL gas-tight bottles initially containing 190 mL of MSM and 20 ml of diluted *Sphagnum* inoculum (from now on referred as S) or 190 mL of MSM and 20 ml of diluted *Sphagnum* + activated sludge (50%/50%) (from now on referred as M). The initial mass of inoculum in each pre-enrichment was 50 g/L. The bottles were closed with butyl septa and plastic caps, and the headspace was flushed with pure O_2 for 15 min in order to eliminate the previous air atmosphere. CH_4 was then supplied at an initial headspace concentration of 195 ± 7 $g\,m^{-3}$ and the bottles were incubated in an orbital shaker MaxQ 4000 from Thermo Scientific, at 200 rpm. Aliquots of 10 ml of the final enrichments S and M at 25 °C were transferred to 1250 mL gas-tight bottles prepared in duplicate as above described. Five enrichment cycles at 25, 30, 37 and 45 °C (each cycle involving a total CH_4 consumption and a complete headspace renewal) were carried out under orbital shaking at 200 rpm in duplicate. The temperature range of these experiments was selected according to the optimum growth temperatures of methanotrophic bacteria, which are in general in the mesophilic range (Hanson and Hanson, 1996). No replacement of the MSM was conducted along the cycles, which entailed that the fifth cycle occurred under nitrogen limiting conditions. This procedure was designed to carry out an enrichment process under high biomass concentrations and low CH_4 aqueous concentrations (30 times lower than in the headspace according to the Henry's law constant for CH_4 of 30 at 25 °C (Sander, 2014)) in order to favor the growth of methanotrophs with low apparent K_m values. In addition, a second enrichment in fresh MSM inoculated with 20 ml of the final cultivation broth resulting from the 5th-cycle enrichment was conducted in duplicate at the different temperatures tested. The concentrations of CH_4, O_2 and CO_2 in the headspace and of TSS in the cultivation broth were periodically monitored in all enrichment experiments.

2.3. Influence of the temperature on CH_4 biodegradation kinetics

The kinetics study was performed in duplicate, batch-wise in gas-tight bottles (1250 mL) initially containing 200 mL of MSM and 7.3 mg of fresh biomass (obtained in the late exponential growth phase from a further culture inoculated with the final culture resulting from the second enrichments conducted in section 2.2). The serum bottles were hermetically closed, and the headspace was saturated with oxygen by flushing pure O_2 for 15 min. CH_4 was added to the headspace with gas tight syringes up to a final concentration of 194 ± 7 $g\,m^{-3}$. The serum bottles were then incubated in an orbital shaker at 25 °C, 30 °C or 37 °C and 200 rpm until methane degradation. CH_4, O_2 and CO_2 concentrations were periodically determined using a GC-TCD. In the case of the cell biomass it was measured as TSS drawing 3 ml from the cultivation broth. The specific growth rate (μ) was calculated from the slope of the logarithm of the biomass concentration *vs.* time. The specific CH_4 biodegradation rates were estimated from the ratio of the specific growth rates and the observed biomass yields in each experiment. The concentration of total nitrogen (TN) in the cultivation broth was also recorded. Neither significant CH_4 biodegradation nor biomass growth was recorded at 45 °C.

2.4. Influence of the temperature on PHAs accumulation under N-limiting conditions

The study was performed in duplicate batch-wise in 1250 mL gas-tight bottles initially containing 200 mL of N-free MSM and fresh biomass from the kinetics test (previously centrifuged and resuspended in N-free MSM). The bottles were closed with butyl septa and plastic caps, flushed with O_2 for 15 min and CH_4 was then supplied at an initial headspace concentration of 177 ± 7 g m^{-3}. The bottles were incubated in an orbital shaker) at 25 °C, 30 °C or 37 °C and 200 rpm until complete CH_4 depletion. In addition, fresh biomass enriched at 25 °C was cultivated under nitrogen starvation conditions at 25, 30 and 37 °C, respectively, to elucidate the role of temperature during the accumulation phase. In this test series, the headspace concentrations of CH_4, O_2 and CO_2, biomass concentration (measured as TSS) in the cultivation broth and the PHB content of the biomass were periodically monitored.

2.5. Analytical procedures

CH_4, O_2 and CO_2 gas concentrations were determined using a Bruker 430 GC-TCD (Palo Alto, USA) endowed with a CP-Molsieve 5A column (15 m × 0.53 μm × 15 μm) and a CP-PoraBOND Q column (25 m × 0.53 μm × 10 μm). The oven, injector and detector temperatures were 45 °C, 150 °C and 200 °C, respectively. Helium was the gas carrier at a flow rate of 13.7 mL min^{-1}. Cell growth was measured as Total Suspended Solids (TSS) according to Standards Methods (American Public Health Association (APHA) et al., 2005). TN concentration was determined following sample filtration (0.45 μm) in a TOC-VCSH attached to a TNM-1 module (Shimadzu, Japan). The bacterial PHB content was measured using a GC–MS (GC System 7820A MSD 5977E, Agilent Technologies, Santa Clara, USA) equipped with a DB-wax column (30 m × 250 μm × 0.25 μm) according to (Frutos et al., 2017).

2.6. DNA extraction, illumina library preparation and 16S rRNA gene sequencing

Amplicon sequencing was developed targeting the 16S V3 and V4 regions (464bp, *Escherichia coli* based coordinates) with the bacterial primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a- A-21, forward and reverse, respectively according to (Klindworth et al., 2013). Universal primers were used instead of specific primers for methanotrophic bacteria in order to account for the entire bacterial population involved in PHA production. Illumina adapter overhang nucleotide sequences were added to the gene-specific sequences according to (López et al., 2018b). Library construction was carried out using the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA). Libraries were then normalized and pooled prior to sequencing. Non-indexed PhiX library (Illumina, San Diego, CA) was used as performance control. Samples containing indexed amplicons were loaded onto the MiSeq reagent cartridge for automated cluster generation paired-end sequencing with a 2 × 300pb paired-end run (MiSeq Reagent kit v3 (MS-102-3001)) according to manufacturer's instructions (Illumina). The amplicon sequencing analysis was carried by the Foundation for the Promotion of Health and Biomedical Research of Valencia Region (FISABIO, Spain).

2.7. 16S rDNA-based taxonomic analysis

Only reads with quality value scores ≥20 in more than 99% of the sequence after demultiplexing and without ambiguous base calls were further analysed. Quality analyses, paired-end reads junction and chimera search were carried out using the prinseq-lite program (Schmieder and Edwards, 2011), the FLASH program (Magoc and Salzberg, 2011) and the USEARCH program (Edgar, 2010). Taxonomic assignments were then conducted using the RDP-Classifier from the Ribosomal Database Project (Cole et al., 2009; Wang et al., 2007). Simpson and Shannon indices were determined using the Vegan library version 2.3e1 (Oksanen et al., 2015). The Krona tool was used to represent relative abundances and confidences within the complex hierarchies of metagenomics classifications (Ondov et al., 2011). Nucleotide sequence dataset obtained in this study has been submitted to NCBI Sequence Read Archive with the bioproject accession PRJNA521405 (https://trace.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA521405&go=go).

2.8. Statistical analyses

All analyses were performed using biomass obtained from two biological replicas for each condition. The specific growth rates, biomass yields, specific CH_4 biodegradation rates, PHAs contents and PHAs produced per unit of CH_4 consumed, were compared pairwise using a single cued *t*-test assuming equal variance. The differences were considered statistically significant for p-values below 0.05. The symbols "*", "**" and "***" were used to indicate values below 0.05, 0.01 and 0.001, respectively, in the figures. In the particular cases where two conditions were not significantly different from each other, their values were grouped and a *t*-test was performed compared to a third condition. Vegan (Oksanen et al., 2015) and stats packages in R (R Development Core Team, 2016) were used for the statistical analyses of the metagenomics data.

3. Theoretical framework

3.1. Stoichiometry of PHA production

PHA production using methane as the sole carbon and energy source requires a biomass formation step under nitrogen excess conditions followed by a PHA accumulation step under nitrogen limiting conditions. This two-stage process can be implemented in two separate units (Fig. S1). The CH_4 and nitrogen required to produce a unit of PHA (disregarding CH_4 maintenance consumption, which is small compared to its consumption associated biomass and PHA formation) will depend on the biomass yield on CH_4 ($Y_{X/CH4}$), the potential PHA content accumulated per unit of biomass ($F_{PHA} = m_{PHA}/X$; X being the initial biomass without PHA accumulation), the PHA yield on CH_4 ($Y_{PHA/CH4}$) and the fraction of nitrogen contained in the biomass before PHA accumulation (F_N). Thus, the production of one unit of PHA requires the previous formation of an amount of biomass X equal to $1/F_{PHA}$, which itself requires a CH_4 supply equal to $X/Y_{X/CH4}$ in the first unit (under nitrogen excess). On the other hand, the amount of CH_4 to be supplied to the second unit (under nitrogen limiting conditions) could be estimated as $1/Y_{PHA/CH4}$. Therefore, the total methane supplies necessary to obtain a mass unit of PHA (m_{CH4}) can be calculated as $1/(F_{PHA}Y_{X/CH4}) + 1/Y_{PHA/CH4}$. Likewise, the nitrogen required per unit of PHA m_N is equal to $F_N/(F_{PHA})$.

3.2. Biodegradation kinetics and gas-liquid CH_4 transfer

Both the biomass formation and PHA accumulation units require the transfer of CH_4 (and oxygen) from the gas emission to the methanotrophic cultivation broth. Under steady state operation, the rate of CH_4 and O_2 mass transfer per unit of reactor volume will be equal to the volumetric rate of microbial consumption of these substrates. The rate of CH_4 mass transport per volume of reactor (R_{CH4}) can be described by the following equation (Bordel et al., 2008) (Eq. (1)):

$$R_{CH4} = \frac{Q_g}{V}\beta_{CH_4}\left(C_{gin} - H_{CH_4}C_L\right) \quad (1)$$

Where C_{gin} and H_{CH4} stand for the inlet concentration and Henry's law constant of CH_4, V the bioreactor volume, Q_g the gas flow rate and C_L the CH_4 aqueous concentration in the cultivation broth. The parameter β_{CH4} ranges between 0 and 1, and depends mainly on the gas-liquid interface area in the reactor as well as the transport properties of the

system (Bordel et al., 2008) (Eq. (2)).

$$\beta_{CH_4} = 1 - exp\left(\frac{-k_L a V}{H_{CH_4} Q_g}\right) \quad (2)$$

Where $k_L a$ represents the volumetric mass transfer coefficient of CH_4 in the bioreactor. The value of the parameter β_{CH4} is equal to the maximum fraction of CH_4 transferrable from the gas emission to the cultivation broth within the reactor. This parameter is independent from the biological parameters of the microbial community present in the cultivation broth. Therefore, it is desirable to operate with β_{CH4} values close to 1. However, low β_{CH4} values are typically recorded for gas substrates with a low aqueous solubility such as CH_4 (e.g the dimensionless Henry constant H_{CH4} is equal to 33.5 at 30 °C (Duan et al., 1992)), which would ultimately limit the performance of PHA production based on CH_4. In this context, several high mass transfer performance bioreactors such as two-phase partitioning, Taylor flow or internal gas recycling bioreactors have been engineered to overcome these limitations (Bordel et al., 2010; García-Pérez et al., 2018; Hernández et al., 2011). However, the kinetic parameters of the methanotrophic community influence the CH_4 mass transport rate since microbial kinetics ultimately determine the steady state liquid concentration (C_L). The specific biomass growth rate of methanotrophs (μ) can be accurately described using the Monod's equation (Eq. (3))

$$\mu = \mu_{max} \frac{C_L}{C_L + K} \quad (3)$$

Where μ_{max} stands for the maximum specific growth rate and K the Monod half-saturation constant. The specific biomass growth rate in the biomass formation unit under steady state is equal to the dilution rate D (= volumetric liquid flow rate supplied to the reactor divided by the reactor volume). Hence, the CH_4 aqueous concentration, C_L, can be determined as a function of the dilution rate (Eq. (4)).

$$C_L = \frac{DK}{\mu^{max} - D} \quad (4)$$

The Monod constant K is equal to the concentration at which the cell growth rate is equal to half its maximum value (μ_{max}). The value of K in methanotrophic cultures typically ranges between 2 and 12 μM. On the other hand, the maximum specific growth rate sets a threshold to the dilution rate of the bioreactor. Thus, the dilution rate should be at least an order of magnitude lower than this critical dilution rate in order to cope with increases in the necessary liquid flow to be processed and to avoid biomass wash-out. Based on equation [1], the fraction (F_{CH4}) of CH_4 in the gas emission transferred to the cultivation broth can be estimated as follows (Eq. (5)):

$$F_{CH4} = \beta_{CH4} \frac{C_{gin} - H_{CH4} C_L}{C_{gin}} \quad (5)$$

In this context, while the parameter β_{CH4} depends only on the gas-liquid mass transfer capacity of the bioreactor, C_L is governed by the liquid dilution rate and the kinetics parameters of the microbial community. Therefore, the fraction F_{CH4} can be regarded as the product of a transport limited term and a biologically limited fraction. This biologically limited term is close to 1 for the typical kinetics and operating parameters prevailing in CH_4 abatement bioreactors, which entails that the impact of the maximum specific biomass growth rate on the overall process performance is small.

4. Results

4.1. Influence of the enrichment temperature on the specific growth rates

A decrease in the specific growth rate of the microbial communities was observed when increasing the selected temperature in the enrichments derived from *Sphagnum* alone at a significant level of $p \leq 0.05$ (Fig. 1). Thus, average specific growth rates of 0.05 ± 0.001 h^{-1}, 0.04 ± 0.004 h^{-1} and 0.02 ± 0.004 h^{-1} were observed at 25, 30 and 37 °C, respectively, in S enrichments. However, the specific growth rates of the microbial communities enriched from the mixed inoculum (M) increased from 0.02 ± 0.002 h^{-1} at 25 °C to 0.033 ± 0.0009 h^{-1} at 37 °C. Interestingly, the specific growth rates of the two M biological replicates enriched at 30 °C differed strongly (0.022 and 0.042 h^{-1}), resulting in no statistical differences with the M enrichments at 25 and 37 °C.

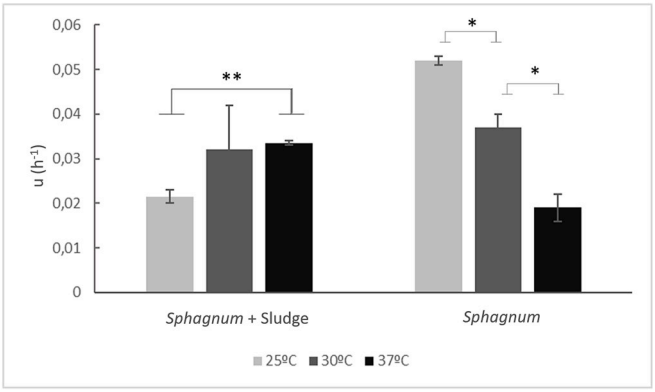

Fig. 1. Influence of the enrichment temperature on the specific growth rates for the communities enriched from *Sphagnum* and *Sphagnum* + activated sludge. For the mixed *Sphagnum* and activated sludge inoculum, error bars show standard errors (n = 2), and the specific growth rate of the biomass enriched at 37 °C was significantly higher than the growth rate at 25 °C (p-value 0.008, n = 2). For *Sphagnum* inoculum, the opposite trend was observed, with decreasing specific growth rates at higher temperatures (p-values of 0.02 between 25 and 30 °C and 0.03 between 30 and 37 °C, n = 2).

4.2. Influence of the enrichment temperature on the specific CH_4 degradation rate

No significant difference was observed between the specific CH_4 biodegradation rates of the biomass enriched from *Sphagnum* at 25 and 30 °C (65.3 ± 5.1, 79.5 ± 7.9 mg-CH_4 h^{-1} g-biomass^{-1}, respectively; error intervals are standard errors with 2 samples) (Fig. 2). However, a significantly lower specific CH_4 biodegradation rate was observed at 37 °C (49.7 ± 3.7 mg-CH_4 h^{-1} g-biomass^{-1}), which correlated with the lower μ recorded at this temperature. On the other hand, the

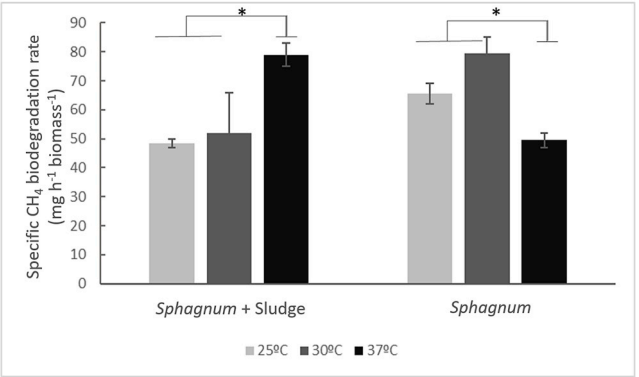

Fig. 2. Influence of the enrichment temperature on the specific CH_4 biodegradation rate for the communities enriched from *Sphagnum* and *Sphagnum* + activated sludge. Error bars are standard errors (n = 2). In both cases, no significant difference is observed between the enrichment at 25 and 30 °C. The biomass enriched at 37 °C showed a significant increase (p-value 0.017; *t*-test for samples of different sizes n = 2, m = 4) for the mixture of *Sphagnum* and activated sludge and a significant decrease (p-value 0.018; *t*-test for samples of different sizes n = 2, m = 4) for *Sphagnum* alone.

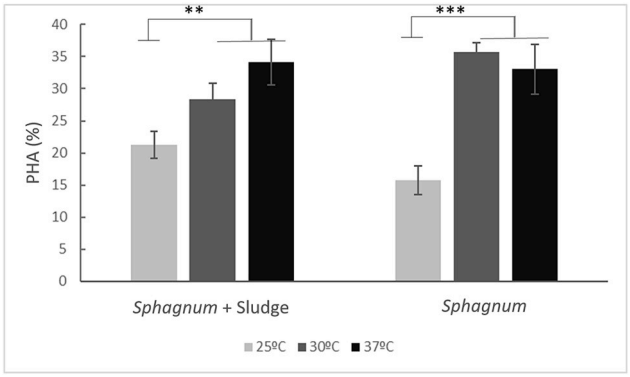

Fig. 3. Influence of the enrichment temperature on the PHA content of the biomass enriched from *Sphagnum* and *Sphagnum* + activated sludge. Error bars represent standard errors (n = 2). The same trend was observed for the mixture of *Sphagnum* and activated sludge and *Sphagnum* alone. Significantly higher PHA contents were observed at 30 and 37 °C compared to 25 °C (p-values of 0.00015 for *Sphagnum* and 0.005 for the mixture; *t*-test for samples of different sizes n = 2, m = 4). The differences between 30 and 37 °C were not statistically significant.

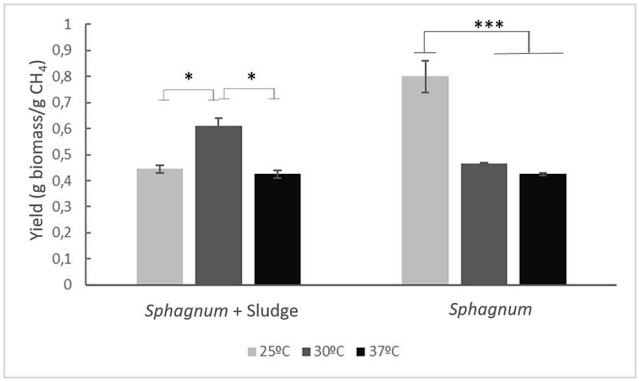

Fig. 4. Influence of the enrichment temperature on the biomass yield on CH_4 from *Sphagnum* and *Sphagnum* + activated sludge. Error bars represent standard errors (n = 2). For the mixture of *Sphagnum* and activated sludge, the biomass enriched at 30 °C showed a significantly higher yield (p-value 0.012; *t*-test for samples of different sizes n = 2, m = 4) compared to the biomass enriched at 25 and 37 °C. For the *Sphagnum* alone a very significant increased yield was observed for the biomass enriched at 25 °C (p-value 0.00097; *t*-test for samples of different sizes n = 2, m = 4).

biomass enriched from the mixed inoculum (M) exhibited lower specific CH_4 biodegradation rates at 25 and 30 °C (48.2 ± 2.5 and 51.7 ± 1.9 mg-CH_4 h^{-1} g-biomass^{-1}, respectively) than at 37 °C (78.9 ± 5.6 mg CH_4 h^{-1} g biomass^{-1}). The differences obtained between the specific CH_4 biodegradation rates of S and M confirmed that the experiments were conducted under non-mass transfer limiting conditions.

4.3. Influence of the enrichment temperature on PHAs content

The content of biopolymers accumulated by the microorganisms of both inocula increased with temperature. Polyhydroxybutyrate was the dominant PHA in all the samples analysed. The biomass enriched at 30 and 37 °C was able to accumulate over 30% of PHA (on a dry weight basis) inside the cells (35.1 ± 0.4% and 33.1 ± 1.3% in S at 30 °C and 37 °C, respectively, and 28.4 ± 1.1% and 34.1 ± 1.3% in M at 30 °C and 37 °C, respectively) under nitrogen limiting conditions, compared to the PHA contents of 15.7 ± 2.4% and 21.2 ± 2.0% in S and M biomass, respectively, at 25 °C (Fig. 3). The mass of PHA per unit of CH_4 consumed was also obtained from experiments conducted under N limiting conditions. Interestingly, no significant difference was observed among the 6 microbial communities enriched, with average values of 0.22 ± 0.05 g-PHA (g-CH_4)$^{-1}$. In this context, this value has been previously reported to be 0.57 g-PHA (g-CH_4)$^{-1}$ for pure cultures of *Methylocystis hirsuta*, which is a high-performance PHA accumulating methanotrophic strain (López et al., 2018a). Similarly, Chidambarampadmavathy et al. (2017), using a consortium enriched from landfill soil and dominated by *Methylosarcina sp.*, obtained a maximum PHB content of 25 mg g biomass^{-1} at 40% of CH_4 in air Zhang et al. (2018) reported accumulations of ~45% of PHB in a mixed culture enriched from sewage sludge under N limitation in the presence of Cu, whereas the absence of Cu decreased PHB synthesis (12%–18%).

4.4. Influence of the enrichment temperature on biomass yield

The biomass enriched from *Sphagnum* at 25 °C showed a yield on CH_4 almost twice as high as the biomass enriched from this inoculum source at the two other temperatures (Fig. 4). This higher yield was also correlated to the higher specific growth rate observed at this temperature. As discussed later, this higher yield might be somehow related to the higher population diversity observed in this sample. The biomass derived from the mixed inoculum at 25 °C did not show yields higher than that enriched at 30 and 37 °C. In contrast, the higher yield on CH_4 was recorded in the biomass enriched at 30 °C.

4.5. Selection of optimal enrichment conditions for PHA accumulation

From an industrial perspective, this study aimed at elucidating the optimal enrichment temperature and inoculum source that potentially result in the most effective usage of raw materials (in this case CH_4 and N). Table 1 depicts the CH_4 and nitrogen required to produce a unit of PHA as a function of the enrichment temperature and inoculum source. The most cost-effective biomass for optimal PHA production would be the enrichment from *Sphagnum* at 30 °C. This biomass required the lowest CH_4 input (10.5 ± 1.4 g CH_4 g PHA^{-1}; equal to that required by the biomass obtained at 30 °C from the mixed inoculum) and also the lowest nitrogen input (0.2 g N g PHA^{-1}).

4.6. Influence of the temperature during the accumulation phase on PHA content

The results herein obtained showed that, independently of the inoculum source, the biomass enriched at 30 and 37 °C led to the highest PHA yields. In the previous experiments in literature, biomass enrichment and PHA synthesis (under nitrogen limitation) were carried out at a constant temperature (García-Pérez et al., 2018; López et al., 2018a, 2018b; Myung et al., 2017; Zhang et al., 2017). In order to elucidate whether these higher PHA yields were the result of the temperature during the enrichment or accumulation phase, the biomass enriched at 25 °C was supplied with CH_4 under nitrogen starvation conditions at 25, 30 and 37 °C. No increase in the PHA content of the biomass enriched at 25 °C was observed at the two highest temperatures. Indeed, a slight decrease (no statistically significant) was found at 30 and 37 °C (Fig. 5).

4.7. Influence of the enrichment temperature on the structure of the microbial communities

The enrichment process (pre-enrichment + 5 growth stages till complete N depletion + 2 growth stages + 1 PHA accumulation stage) resulted in the dominance of the genus *Methylocystis* (with percentages close to 30%, Fig. S2) in the enriched communities regardless of the inoculum source and temperatures. Members of this genus, which was almost absent in the initial inocula, are capable of synthetizing PHA under nitrogen deprivation conditions (López et al., 2018a). The biomass enriched from *Sphagnum* contained a low number of other methanotrophs and methylotrophs (~1% of *Methylobacterium* at 30 and

Table 1
Methane and nitrogen requirements and biomass production per gram of PHA produced.

	Sphagnum			*Sphagnum* + Activated sludge		
	25 °C	30 °C	37 °C	25 °C	30 °C	37 °C
Biomass per gram of PHA (g)	6.3 ± 0.6	2.8 ± 0.04	3 ± 0.05	4.7 ± 0.2	3.5 ± 0.2	2.9 ± 0.1
CH_4 growth phase (g)	4.5 ± 1	4.5 ± 1	4.5 ± 1	4.5 ± 1	4.5 ± 1	4.5 ± 1
CH_4 accumulation phase (g)	7.9 ± 2.4	6 ± 0.4	7 ± 1.3	10.5 ± 2	5.8 ± 1	6.8 ± 1.4
Total CH_4 per gram of PHA (g)	12.5 ± 3.4	10.5 ± 1.4	11.6 ± 2.3	15 ± 3	10.3 ± 2	11.4 ± 2.4
Nitrogen per gram of PHA (g)	0.5 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0

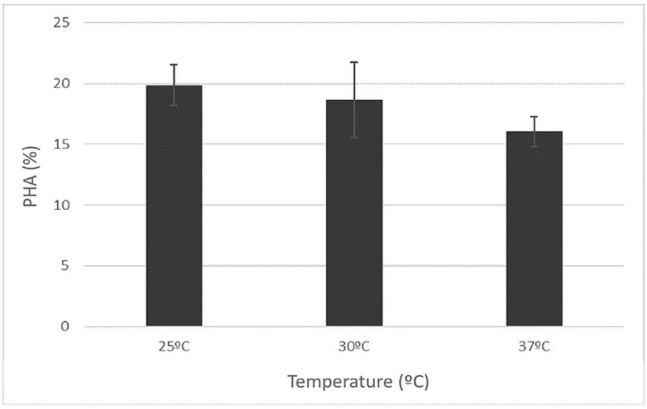

Fig. 5. Influence of the temperature during the accumulation phase on the PHB content of the biomass enriched from *Sphagnum* at 25 °C under nitrogen starvation at 25, 30 and 37 °C, respectively. Error bars are standard errors (n = 2).

37 °C, and 2% of *Methylophylus* at 25 °C). On the other hand, the biomass enriched from the mixed *Sphagnum* and activated sludge inoculum contained significant amounts of methylotrophs such as *Methylophilus* (17% of the community enriched at 25 °C), *Methylobacterium* (5% of the community enriched at 30 °C) and methanotrophs such as *Methyloparacocus* (9% of the community enriched at 30 °C). These methanotrophs and methylotrophs different from the genus *Methylocystis* were less abundant in the community enriched at 37 °C (1% of *Methylobacterium*). The results here obtained revealed that temperature was a strong selective pressure for the enrichment of PHA accumulating bacteria regardless of the inoculum.

None of the characteristics of the microbial communities enriched (specific growth rate, PHA accumulation etc.) seemed to be explained by changes in the overall microbial composition. For example, the enrichments conducted at 30 and 37 °C, which resulted in higher PHA accumulations, did not contain a higher percentage of *Methylocystis* than those conducted at 25 °C.

5. Discussion

5.1. Effect of the enrichment conditions on the microbial community profile

This study aimed at assessing both the feasibility of enriching PHA accumulating methanotrophic consortia from *Sphagnum* mosses-based inocula (based on their high inherent abundance in type II methanotrophs) and the influence of the temperature on the characteristics of the communities enriched. The prolonged exposure of the inocula to methane as the only carbon and energy source (following the described enrichment protocol) allowed to enrich microbial consortia where the genus *Methylocystis* was the most abundant (which represented approx. 30% of the total microbial population) regardless of the enrichment temperature. *Methylocystis* are known to accumulate PHA, however no selective pressure aiming to favor PHA accumulating organisms (such as alternate cycles of nitrogen starvation) was specifically imposed during the enrichment process. The specific growth rates of the consortia enriched were up to two folds higher than those previously reported for pure cultures of *Methylocystis hirsuta* (López et al., 2018a) (0.021 h^{-1}), likely due to the symbiotic effects with other microbial populations. In this context, the purer a culture is, the higher are the restrictions to grow under environmental stress factors, while a higher population richness and diversity promotes higher resilience and therefore, better bio-product recovery (Cabrol et al., 2012). Moreover, adaptive laboratory evolution could be also carried out to gain insights into the adaptive changes that experience microbial populations during long-term selection: better PHA productivity and higher methane degradation rates (Dragosits and Mattanovich, 2013).

5.2. Specific growth rate and microbial CH_4 bioconversion

In addition, the theoretical framework developed in section 3 reveals that a microbial community with a maximum specific growth rate of 0.02 h^{-1} (the minimum recorded in this study) treating a gas emissions with typical CH_4 inlet concentration of 6% (equivalent to an equilibrium concentration of 0.0024 M in the aqueous phase) will eventually support a CH_4 removal of 99.2% in the bioreactor (under no mass transfer limitations), while a biomass with a maximum specific growth rate of 0.05 h^{-1} (the maximum recorded in this study) will support a potential CH_4 removal of 99.7%. These estimations clearly show that the maximum specific growth rate of the microbial community enriched does not represent a key parameter in suspended-growth CH_4 bioconversion processes operated under continuous mode. The scenario would be different if the process was performed batch-wise, where higher maximum specific growth rates would result in shorter production times.

The biomass yields on methane here recorded were of the same order as those of pure cultures of *Methylocystis*, with the exception of the consortium enriched from the inoculum S at 25 °C, which showed a biomass yield almost twice as large as that of a pure *Methylocystis hirsuta* cultures. It can be hypothesized that this unusually high yield (Y_{X/CH_4} = 0.8) was supported by the high microbial diversity of this particular enrichment (H = 2.84). A weak correlation between the biomass yield and the microbial diversity was also observed (R = 0.764 and a p-value = 0.109), which has been previously described in literature (Louis et al., 2016).

5.3. Effect of temperature on PHA accumulation

Since temperature has been identified as an important factor that modifies PHA accumulation in other heterotrophic bacteria (Myshkina et al., 2008), this study aimed at assessing the optimal temperature conditions during culture enrichment in order to increase the production of PHA using type II methanotrophs. Culture enrichment at 30 and 37 °C resulted in consortia able to accumulate significantly higher amounts of PHA under nitrogen starvation (30% of the total microbial biomass) than those enriched at 25 °C. These values were low in comparison to the PHA contents of 40–47% (wt) reported by Zhang et al. (2018). This result was probably due to the high copper concentration used by Zhang et al. (2018) during culture enrichment, which increased PHA accumulation by a factor of 2.5. Moderate concentrations of

copper (as typically found in urban wastewater treatment plants) were used in this study to avoid the effect of additional variables on the results obtained (Zhang et al., 2018). Nevertheless, the fact that only 30% of the DNA of the enriched consortia corresponded to bacteria of the genus *Methylocystis* (the only described PHA accumulating organism identified in these consortia) suggested that the strains of this genus in these specific enrichments could eventually contain up to 70% of PHA. In this regard, under nutrient-limited conditions *Methylocystis, Methylosinus* and *Methylocella*, which are considered the main methanotrophic PHA producers to date, are able to achieve PHA accumulations ranging from 20 to 50% (wt) (García-Pérez et al., 2018; Pieja et al., 2012; Zhang et al., 2017) values that are lower than the ones suggest values that were lower than those reported in literature under comparable conditions.

The fact that the results obtained for the PHA content of the microbial communities enriched at different temperatures were just due to the temperature prevailing during the accumulation phase was discarded by performing PHA accumulation experiments at 25, 30 and 37 °C with biomass enriched from *Sphagnum* at 25 °C. No enhancement in PHA accumulation was observed at 30 and 37 °C, which confirmed that culture enrichment at higher temperatures plays a key role on the selection of more efficient PHA accumulators. However, the mechanisms underlying the superior accumulation of PHA in communities enriched at higher temperatures remains unknown. In this context, it has been speculated that temperature acts as an environmental stress analogous to nitrogen depletion and triggers PHA accumulation. However, only trace levels of PHA were observed at 30 or 37 °C in cultures grown under nitrogen sufficient conditions and there was not a direct influence of temperature on PHA accumulation under nitrogen limiting conditions during the accumulation phase. At this point it can be speculated that some molecular mechanisms involved in heat shock resistance might also enable methanotrophs to accumulate higher PHA contents. Finally, it should be highlighted that lower temperatures increase CH_4 mass transfer from the gas to the microbial community as a result of a decrease in the Henry's law constant, which directly impact on the gas-liquid concentration gradient and could influence the specific growth rates and PHA content of the biomass. However, the theoretically enhanced CH_4 mass transfer at lower temperatures did not increase the PHA content of the microbial communities enriched.

6. Conclusions

This study confirmed the key role that the enrichment temperature plays on microbial PHA production from CH_4, culture enrichment at high temperatures (30 and 37 °C) resulted in a superior PHA accumulation compared to the biomass enriched at 25 °C. This finding suggests that temperature has an important effect on PHA accumulation and could enhance the implementation of PHA cell factories.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibiod.2019.04.004.

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